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Yvonne M. Janssen
University of Vermont


Aaron Barchowsky
Dartmouth College

Melinda Treadwell
Dartmouth College

Kevin E. Driscoll
Procter and Gamble

B T. Mossman
University of Vermont

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Asbestos induces nuclear factor κ B (NF- κ B) DNA-binding activity and NF- κ B-dependent gene expression in tracheal epithelial cells

(lung disease/lung cancer/protooncogenes/transcription factors)

YVONNE M. W. JANSSEN*, AARON BARCHOWSKY†, MELINDA TREADWELL†, KEVIN E. DRISCOLL‡, AND BROOKE T. MOSSMAN*

*Department of Pathology, University of Vermont College of Medicine, Medical Alumni Building, Burlington, VT 05405; †Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755; and ‡Miami Valley Laboratories, Procter and Gamble, Cincinnati, OH 45239

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ABSTRACT Nuclear factor κ B (NF- κ B) is a transcription factor regulating expression of genes intrinsic to inflammation and cell proliferation—features of asbestos-associated diseases. In studies here, crocidolite asbestos caused protracted and dose-responsive increases in proteins binding to nuclear NF- κ B-binding DNA elements in hamster tracheal epithelial (HTE) cells. This binding was modulated by cellular glutathione levels. Antibodies recognizing p65 and p50 protein members of the NF- κ B family revealed these proteins in two of the DNA complexes. Transient transfection assays with a construct containing six NF- κ B-binding DNA consensus sites linked to a luciferase reporter gene indicated that asbestos induced transcriptional activation of NF- κ B-dependent genes, an observation that was confirmed by Northern blot analyses for *c-myc* mRNA levels in HTE cells. Studies suggest that NF- κ B induction by asbestos is a key event in regulation of multiple genes involved in the pathogenesis of asbestos-related lung cancers.

Exposure to asbestos is associated with the development of pulmonary fibrosis, lung cancer (bronchogenic carcinoma), and malignant mesothelioma (1). Although mechanisms of asbestos-induced diseases are unclear (2, 3), chronic inflammation and cell proliferation are common features in the pathogenesis of both fibrotic and malignant lesions. Work to date suggests that asbestos may interact with target cells of disease through multiple mechanisms involving active oxygen species and/or elaboration of growth factors (2–6). In studies here, we examined whether asbestos caused nuclear translocation and DNA binding of nuclear factor κ B (NF- κ B), a highly regulated transcription factor linked to activation of a number of genes that contain NF- κ B-binding cis-regulatory elements in their promoter or intronic regions. These genes, which include genes encoding various interleukins and nitric oxide synthase and the protooncogene *c-myc*, may be intrinsic to cell proliferation and inflammation (7–12), features of asbestos-induced pulmonary diseases (1, 2).

Proteins encoded by multiple members of the *Rel* family of genes bind to NF- κ B-binding sequences in DNA as homodimers or as heterodimers with p65. Expression of the *Rel* family of genes is transcriptionally regulated, and steady-state levels of both p65 and p105 mRNA increase following exposure to cytokines (9, 10). Moreover, binding of the NF- κ B transcription factor to recognition sequences in DNA is also subject to complex regulation at the posttranslational level that involves phosphorylation and proteolysis of the inhibitory protein, I κ B, to unmask the nuclear translocation signal of preexisting cytoplasmic NF- κ B complexes (7, 13). A number of studies have shown that nuclear retention and DNA binding of NF- κ B protein complexes are increased following exposure of

various cell types to a variety of extracellular stimuli that include oxidative stress (14), hypoxia (8, 15), inflammatory cytokines (16), and ultraviolet (UV) (17) or ionizing radiation (18). Since oxidants are involved in the cytotoxic effects of asbestos in various target cells *in vitro* (3–5) and the development of disease in a rat inhalation model (19), we hypothesized that crocidolite asbestos, the most pathogenic type of asbestos fiber (1, 2), would cause increases in DNA binding of NF- κ B family members and transcriptional activation of NF- κ B-dependent genes. Using hamster tracheal epithelial (HTE) cells, a progenitor cell type of bronchogenic carcinoma, we demonstrate that exposure to asbestos causes dose-responsive, protracted increases in binding to the NF- κ B-binding DNA sequence (“NF- κ B consensus sequence”) which may be modulated by the antioxidant *N*-acetyl-L-cysteine (NAC). In transient transfection studies using constructs with NF- κ B consensus sites, we also show transcriptional activation of NF- κ B-dependent genes by asbestos. In addition, we demonstrate increased gene expression of *c-myc*, an early response gene containing an NF- κ B-binding cis-regulatory element in its promoter region, by asbestos in HTE cells. Data suggest that persistent activation of NF- κ B by asbestos may contribute to the induction of multiple genes that are critical to the pathogenesis of asbestos-associated diseases.

METHODS

Cell Culture and Exposure to Test Agents. A line of HTE cells previously isolated and characterized in our laboratory (20) was propagated in Ham’s F-12 medium (GIBCO) containing 50 units of penicillin and 50 μ g of streptomycin per ml and 10% (vol/vol) newborn bovine serum. Cells were grown to confluency, and the growth medium was replaced with medium containing 2% serum for 24 hr before addition of test agents.

National Institute of Environmental Health Sciences processed crocidolite asbestos [(Na₂(Fe^{III})₂(Fe^{II})₃Si₈O₂₂(OH)₂] was obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Littleton, CO). Asbestos was added directly to the medium at nontoxic concentrations of 1.25 or 5 μ g/cm² of dish as determined previously (21). Lipopolysaccharide (LPS; *Escherichia coli* 026:B6; Sigma) was used as a positive control for induction of NF- κ B-dependent gene expression at a concentration of 100 ng/ml (11). In some experiments, cells were incubated with NAC (10 mM; Sigma) for 18 hr prior to an 8-hr addition of asbestos. NAC was dissolved in Hanks’ balanced salt solution, and the pH was adjusted to 7.4 with NaOH before addition to cells (22).

Gel Mobility-Shift Experiments. At selected time periods after exposure to test agents, cells were harvested for preparation of nuclear extracts as described by Staal *et al.* (23). Gel

mobility-shift assays were performed by using 2–4 μg of nuclear protein as determined by the Bradford method (24). DNA binding buffer contained 40 mM Hepes buffer, 4% Ficoll 400, 200 ng of poly(dI)·(dC) per μL , 1 mM MgCl_2 , 0.1 mM dithiothreitol, and 0.175 pmol of a ^{32}P -end-labeled double-stranded oligonucleotide (25) containing a consensus NF- κB site (Promega). Protein extracts were incubated in DNA binding buffer for 20 min at room temperature prior to electrophoresis on a 5% polyacrylamide gel (26). Gels were dried and visualized by exposure to Kodak X-Omat film. Radioactivity in retarded binding complexes was quantitated on a phosphorescence imager (Bio-Rad). To determine the specificity of the gel-shift complexes, a 40-fold molar excess of unlabeled NF- κB -binding oligonucleotide or an unlabeled oligonucleotide containing a consensus AP-1-binding sequence (FSE,26) was included in the binding reactions. In addition, antibodies recognizing p50 or p65 members of the NF- κB family [SC-109 and SC-114, respectively (1 mg/ml); Santa Cruz Biotechnology, Santa Cruz, CA] were used to identify the proteins present in the retarded complexes. For these studies, nuclear proteins were incubated in DNA-binding buffer for 20 min, and subsequently 2 μL of antibody was added for an additional 30 min at room temperature prior to resolution of complexes.

Transfection Studies. To determine whether asbestos caused transcriptional activation of NF- κB -dependent genes, transient transfection studies were performed in HTE cells by using the calcium phosphate coprecipitation technique (27). A plasmid construct containing six NF- κB -binding DNA consensus sites linked to a luciferase reporter gene (6X κB -tk-Luc) was utilized. The empty cassette (tk-36-Luc) was used as a negative control. Both constructs were generously provided by P. A. Baeuerle (Biochemisches Institut, Albert-Ludwigs-Universität, Freiburg, Germany). The plasmid pSV- βgal (Promega) was cotransfected with constructs described above to enable normalization of luciferase activity to β -galactosidase activity (25), providing an estimation of differences between transfection efficiencies from dish to dish. After incubation of HTE cells with the calcium phosphate–DNA precipitate for 4 hr, cells were washed once in Hanks' balanced salt solution and allowed to recover overnight in culture medium. Cells were then switched to 2% serum-containing medium for 8 hr. Asbestos or LPS was added at concentrations indicated above for 4 or 16 hr, at which times cells were harvested in lysis buffer (Promega) for determination of luciferase (Promega) and β -galactosidase activities and protein content. Luciferase activity is expressed in relative units after normalization to β -galactosidase activity and protein.

Northern Blots. To determine whether genes with NF- κB -binding cis-regulatory regions in their promoter or intronic regions were induced in HTE cells after exposure to asbestos, steady-state mRNA levels of *c-myc*, a candidate gene regulated by NF- κB (12), were measured in HTE cells. HTE cells were exposed to 2.5 μg of crocidolite asbestos per cm^2 for 2, 4, 8, or 24 hr, and total RNA was extracted and prepared for Northern blot hybridization (28). *c-myc* cDNA (obtained from Jen-Fu Chiu, Department of Biochemistry, University of Vermont) was labeled with [α - ^{32}P]dATP by random hexamer priming. Hybridization signals were quantified directly by phosphor imaging or by densitometric analysis of autoradiographs with a Microscan densitometer (Technology Resources, Nashville). Subsequent hybridization of blots with a cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (21), revealed <15% variability among lanes (data not shown).

Statistical Analyses. Data were analyzed by ANOVA using Duncan's procedure to correct for multiple comparisons. Data from Northern blots were also examined by linear trend analysis.

RESULTS

To determine whether asbestos causes activation of NF- κB in target cells of lung cancers, we used gel mobility-shift assays to demonstrate binding of nuclear proteins to the NF- κB -binding consensus DNA sequence. We first assessed patterns of DNA binding induced by asbestos in HTE cells as representative of a progenitor-cell-type of bronchogenic carcinoma. Fig. 1 shows that multiple gel-shift complexes occur—patterns also observed in other models (29–31). A 40-fold molar excess of unlabeled NF- κB competitively blocked binding of all complexes (Fig. 1 *Upper*, lane 2), whereas unlabeled AP-1 did not (Fig. 1 *Upper*, lane 3), confirming the specificity of binding to the NF- κB consensus sequence. An antibody against p50 (Fig. 1 *Lower*, lane 3) diminished both of the gel-shift complexes, indicating its presence in both complexes. In contrast, the antibody recognizing p65 (Fig. 1 *Lower*, lane 2) diminished only the upper complex showing that it was comprised of both p65 and p50 proteins.

The time course of complex formation in HTE cells exposed to 1.25 or 5 $\mu\text{g}/\text{cm}^2$ of crocidolite asbestos is shown in Fig. 2. Complexes containing p50 and p50–p65 heterodimers increased in a dosage-dependent fashion after exposure to asbestos for as long as 24 hr. The extent of asbestos-induced increases were more pronounced in the p65–p50 complex, as significant increases were observed after 4 and 24 hr of exposure ($P < 0.05$). In contrast, asbestos caused smaller increases in the p50 complex that were not statistically significant. Since asbestos fibers at concentrations used here cause

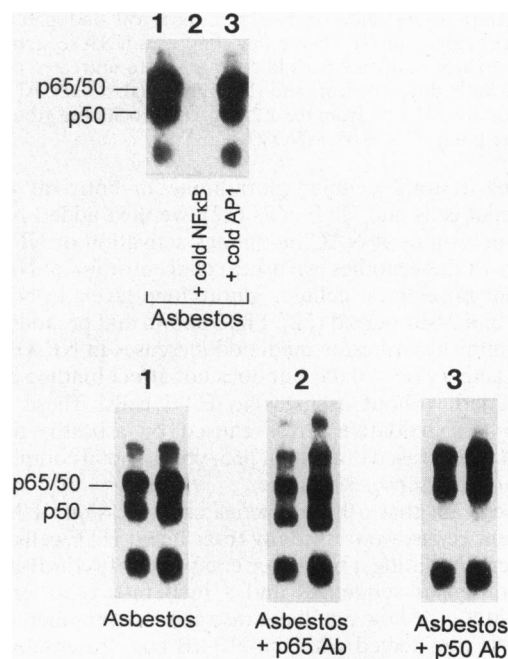


FIG. 1. Specificity of gel-shift complexes in HTE cells. (*Upper*) Nuclear proteins from asbestos-exposed HTE cells (lane 1) were incubated in the presence of a 40-fold molar excess of unlabeled NF- κB -binding oligonucleotide (lane 2) or a 40-fold molar excess of an oligonucleotide containing an AP-1 consensus binding site (oligonucleotide, FSE,26) (lane 3) and then were used in gel-shift assays with a ^{32}P -end-labeled oligonucleotide containing a consensus NF- κB -binding site. Competitive inhibition of both p65–p50 and p50 complexes occurs in the presence of nonradioactive (cold) NF- κB -binding oligonucleotide but not in the presence of nonradioactive AP-1-binding oligonucleotide. (*Lower*) Modification of gel-shift complexes by antibodies recognizing p65 and p50 protein members of the NF- κB family. Nuclear extracts from asbestos-exposed HTE cells (lane 1) were incubated in the presence of antibodies recognizing p65 (lane 2) or p50 (lane 3). p65 and p50 are present in the upper complex, whereas p50 is present in the lower complex, as indicated.

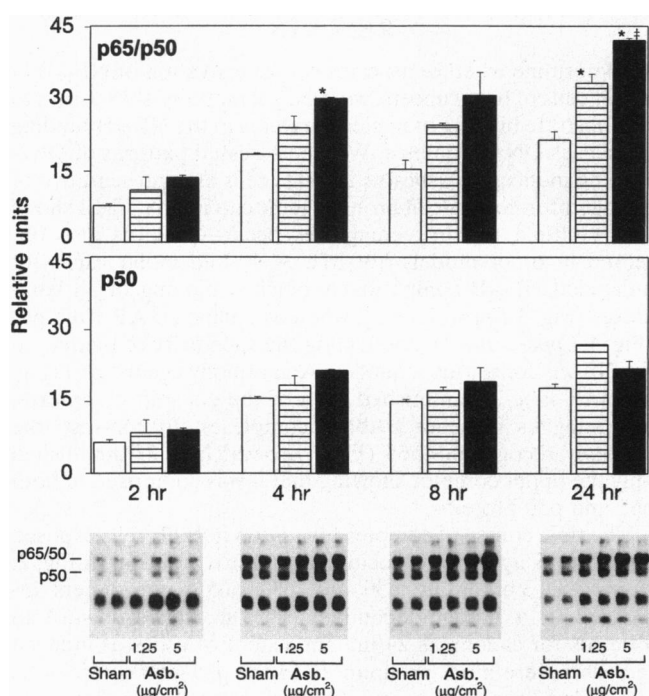


FIG. 2. Asbestos increases binding activity to the NF- κ B consensus sequence in HTE cells. Cells were exposed to crocidolite asbestos (Asb.) at concentrations of 1.25 or 5 μ g/cm² and harvested for preparation of nuclear extracts after 2, 4, 8, and 24 hr of exposure. Gel mobility-shift assays show increases in p65-p50 and p50 protein complexes binding the NF- κ B-binding consensus DNA sequence after exposure to crocidolite asbestos in comparison to untreated controls. *, Significantly different from the sham group ($P < 0.05$; ANOVA); †, significantly different from the 1.25 μ g/cm² crocidolite group at the same time point ($P < 0.05$; ANOVA).

decreases in total cellular glutathione in both rat pleural mesothelial cells and HTE cells (22), we next added NAC to cells to determine if NAC modulates activation of NF- κ B by asbestos. In these studies, we used a concentration of NAC (10 mM) that raises total cellular glutathione levels in both cell types over a 24-hr period (22). Fig. 3 shows that preaddition of NAC diminishes asbestos-mediated increases in NF- κ B DNA binding activity ($P < 0.05$) but does not affect binding activity when added without asbestos to HTE cells. These results indicate that oxidative stress caused by asbestos may be involved in increased binding of p65-p50 and p50 complexes to the NF- κ B consensus sequence.

To ascertain that asbestos activates expression of NF- κ B-dependent genes, we transiently transfected HTE cells with a construct containing a promoter composed of NF- κ B-binding DNA consensus sequences and a luciferase reporter gene. Results in Fig. 4 show that luciferase activities are increased in HTE cells transfected with the NF- κ B consensus sequence-luciferase gene construct (6X κ B-tk-Luc) (Fig. 4 *Upper*) and exposed to LPS (100 ng/ml) or crocidolite (5 μ g/cm²). No alterations were observed in HTE cells transfected with the tk-36-Luc empty vector construct (Fig. 4 *Lower*). LPS caused dramatic increases ($P < 0.05$) in transcriptional activation of the luciferase gene by 4 and 16 hr, whereas significant increases ($P < 0.05$) in crocidolite-induced responses were restricted to 16 hr. This time frame is consistent with the protracted effects of asbestos on cytotoxicity and proliferation in cultured cells (5, 21, 22, 26, 32).

Since *c-myc* is a candidate "early-response" gene activated by NF- κ B (12, 33), we next examined whether *c-myc* mRNA levels were increased in HTE cells after exposure to asbestos. We used concentrations of crocidolite (2.5 μ g/cm² dish) associated with maximal induction of another early-response

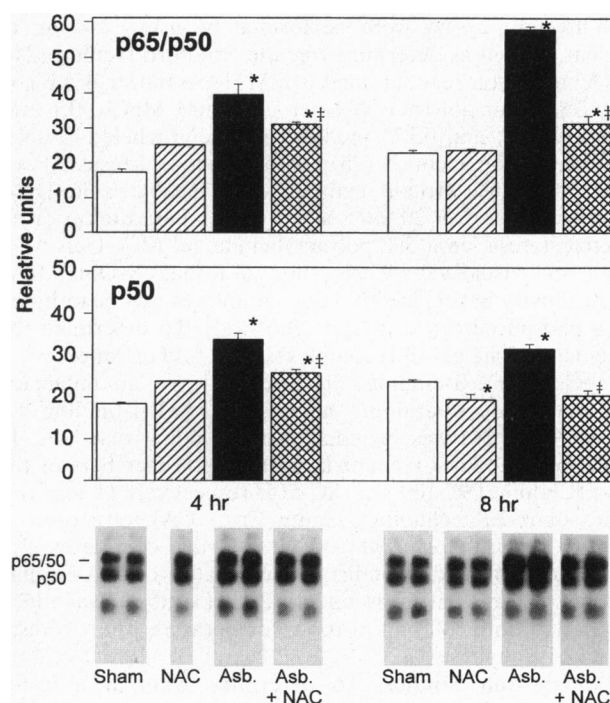


FIG. 3. NAC ameliorates asbestos (Asb.)-mediated increases in binding to the NF- κ B consensus sequence in HTE cells. Note the increases in p65-p50 and p50 complexes in HTE cells after 4 and 8 hr of exposure (*, $P < 0.05$; ANOVA) and significant decreases in asbestos-induced responses after pretreatment with 10 mM NAC (†, $P < 0.05$; ANOVA) when compared with the asbestos-exposed group at the same time point.

gene—i.e., *c-jun*—in this cell type (21, 22, 26). Results in Fig. 5 show that asbestos causes persistent increases in gene expression of *c-myc* that become statistically significant ($P < 0.05$) after 4 hr of exposure and increase over time ($P < 0.001$ by linear trend analysis).

DISCUSSION

Asbestos-associated pulmonary diseases that result from exposure to high airborne concentrations in occupational settings may not become clinically apparent until decades after initial exposure to fibers, and the prognosis is often poor (1–3). Preventive and therapeutic approaches to treatment of fibrosis and malignancies have been hampered by a lack of mechanistic knowledge as to how asbestos fibers activate genes that may be critical to the initiation and development of these lesions. Isolated epithelial cells from the respiratory tract serve as a model system to elucidate the early molecular events triggered by asbestos fibers that may contribute to asbestos-induced bronchogenic carcinoma (20). In studies here, we investigated in this cell type activation by asbestos of NF- κ B, a transcription factor involved in activation of genes that are involved in cell proliferation and inflammation.

The present studies demonstrate increases in binding of p50 and p65-p50 protein complexes to the NF- κ B-binding DNA consensus sequence in HTE cells exposed to asbestos. Amelioration of DNA binding activity by preexposure to NAC suggests that oxidants or alterations in redox status caused by asbestos (22) may contribute to activation of NF- κ B. These results lend further credence to the link between oxidative damage caused by asbestos and the development of pulmonary diseases (19, 34). In other model systems, NAC also inhibits cytokine or oxidant-mediated increases in binding to the NF- κ B-binding DNA consensus sequence (23). Our studies and those of others using a diversity of oxidant stresses support

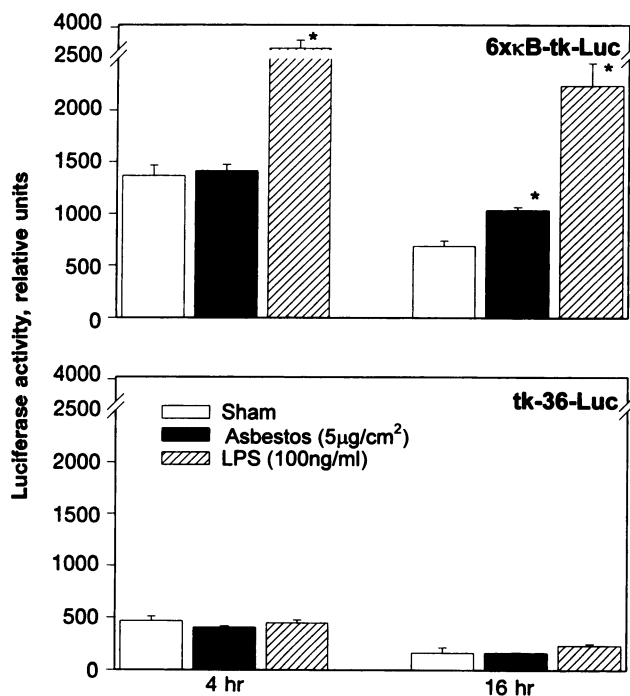


FIG. 4. Transient transfection assays using a construct containing six NF- κ B-binding DNA consensus sequences and a luciferase reporter gene (6X κ B-tk-Luc) (Upper) and the empty cassette control (tk-36-Luc) (Lower). Exposure to the positive control LPS at 100 ng/ml for 4 or 16 hr or to crocidolite asbestos at 5 μ g/cm² for 16 hr resulted in significant increases in luciferase activity in HTE cells transfected with the 6X κ B-tk-Luc construct (*, $P < 0.05$) in comparison to sham controls at the same time point. No alterations were observed in cells transfected with the empty cassette control. Luciferase units are normalized to β -galactosidase activity and protein values, and results are expressed as relative units.

the hypothesis that NF- κ B is an oxidant-sensitive transcription factor (14, 35).

The results of transient transfection experiments using constructs with NF- κ B consensus binding sites indicate that asbestos has the potential to transcriptionally activate a number of NF- κ B-dependent genes integral to immune and inflammatory responses as well as cell proliferation. These observations are strengthened by our experiments showing asbestos-induced mRNA levels of the protooncogene, *c-myc*, an NF- κ B-regulated early-response gene intrinsic to altered cell proliferation and carcinogenesis (12). The protracted dose-dependent induction of *c-myc* becomes more significant ($P < 0.001$) over time, which is in contrast to more rapid and transient increases reported in other cell types stimulated with phorbol 12-myristate 13-acetate, other soluble promoting agents, or carcinogens (36). The prolonged induction of *c-myc* gene expression by asbestos fibers may reflect the time period necessary for fibers to contact cells and be internalized (37). Alternatively, generation of oxidants by intracellular fibers (4, 5, 38) may have to reach critical levels over time to alter the redox state of the cell and allow NF- κ B-dependent induction of *c-myc*, as suggested by our studies using NAC.

The carcinogenicity of asbestos fibers has been linked to their geometry, length (i.e., longer, thinner fibers), and durability (1–3). Based on observations in *in vitro* studies where asbestos fibers were added directly to proliferating embryonic fibroblasts, it has been suggested that fibers physically interact with DNA after penetration of the nuclear membrane during mitosis (39). This phenomenon would be unlikely *in vivo*, as normal cell division in epithelial and mesothelial cells, occurring *in situ* in continuous monolayers connected by junctional complexes, is infrequent. A more plausible scenario suggested

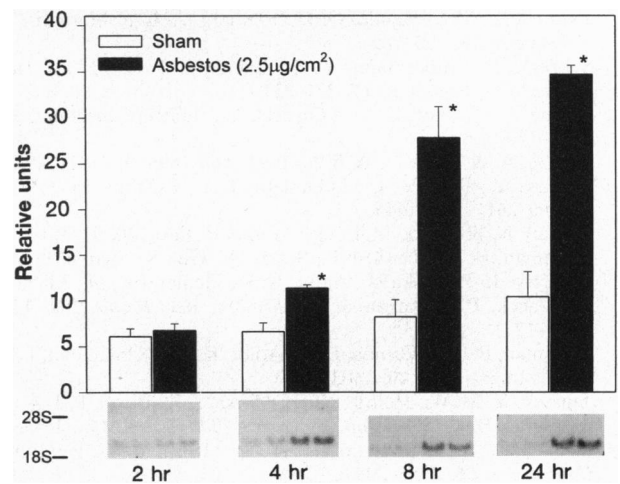


FIG. 5. Northern blot analyses showing the time course of induction of *c-myc* in HTE cells exposed to crocidolite asbestos at 2.5 μ g/cm². After 2, 4, 8, or 24 hr of exposure, total RNA was extracted for Northern blot analyses. Hybridization signals on blots from the 2- and 4-hr time points were quantitated by densitometry, whereas blots from the 8- and 24-hr time points were analyzed with a phosphor imager (*, $P < 0.05$; ANOVA) in comparison to untreated controls from the same time point. In crocidolite-exposed cells, *c-myc* mRNA levels increased over time ($P < 0.001$).

by our data is the persistent stimulation by asbestos of cell signaling cascades leading to activation of transcription factors such as NF- κ B. Demonstration that NAC is inhibitory suggests that these events may be triggered by oxidants liberated from fibers directly or during phagocytosis by HTE cells.

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